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Report

Glypican LON-2 Is a Conserved Negative Regulator of BMP-like Signaling in *Caenorhabditis elegans*

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Summary

Bone morphogenetic protein (BMP) pathways are required for a wide variety of developmental and homeostatic decisions, and mutations in signaling components are associated with several diseases. An important aspect of BMP control is the extracellular regulation of these pathways. We show that LON-2 negatively regulates a BMP-like signaling pathway that controls body length in *C. elegans*. *lon-2* acts genetically upstream of the BMP-like gene *dbl-1*, and loss of *lon-2* function results in animals that are longer than normal. LON-2 is a conserved member of the glypican family of heparan sulfate proteoglycans, a family with several members known to regulate growth-factor signaling in many organisms. LON-2 is functionally conserved because the *Drosophila* glypican gene *dally* rescues the *lon-2(lf)* body-size defect. We show that the LON-2 protein binds BMP2 in vitro, and a mutant variation of LON-2 found in *lon-2(e2140)* animals diminishes this interaction. We propose that LON-2 binding to DBL-1 negatively regulates this pathway in *C. elegans* by attenuating ligand-receptor interactions. This is the first report of a glypican directly interacting with a growth-factor pathway in *C. elegans* and provides a mechanistic model for glypican regulation of growth-factor pathways.

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Results and Discussion

lon-2 Is a Negative Upstream Regulator of the *dbl-1* Signaling Pathway

The nematode *C. elegans* is an established model system for studying the genetic and molecular regulation of BMP signaling [1]. In *C. elegans*, a conserved BMP-like pathway controls body size and male tail morphology (the Sma/Mab pathway). Reducing pathway activity results in animals that are about 55%–85% wild-type length, and males have defective spicules (copulatory structures) and fused sensory rays (Table 1 [2, 3]). Overexpression of BMP-like *dbl-1* results in long animals [2, 3]. Mutations in *lon-2* produce the *dbl-1* overexpression body-size phenotype; animals are 14%–26% longer than the wild-type, with no statistical difference between *lon-2* mutant strains (Table 2; Figures 1A and 1B). To explore the relationship between *lon-2* and the *dbl-1* pathway, we performed epistasis tests with *lon-2(e678)* and mutant alleles of *dbl-1* pathway genes. Genetically, *lon-2* acts upstream of the ligand *dbl-1* and a receptor, *sma-6* (Table 1). Mutations in *lon-1*, a known target gene of the DBL-1 pathway, also result in long animals [4, 5]. Animals with no functional LON-1 and that overexpress *dbl-1* are longer than single-mutant animals [2, 4]. We asked whether loss of *lon-2* expression would have results similar to those of *dbl-1* overexpression in the *lon-1(lf)* background. *lon-1(wk50);lon-2(e678)* animals are also longer than either single-mutant animals (Table 3). Therefore, losing the function of *lon-2* acts like overexpressing *dbl-1*. These results also support the models that body size depends on DBL-1 signal dosage and that the DBL-1 signaling pathway does not act exclusively through *lon-1*.

dbl-1 pathway mutant males also frequently have tail defects [3]. We examined *lon-2(e678)* male tails to see whether *lon-2* has an effect on male tail rays or spicules. We observed no differences in *lon-2(lf)* males compared to wild-type males (data not shown). Unlike Smad mutant males, *lon-2(e678)* males mate normally [6]. Overexpression of *lon-2(+)* also has no effect on the male tail rays or spicules (data not shown). One explanation for this result is that *lon-2* may not function in the male tail. Studies of downstream targets of the *dbl-1* pathway (*lon-1*, *mab-21*, and *mab-23*) have shown that body size and male tail development are separable [2, 4, 5, 7]. Alternatively, dosage effects may be different in the tail than in the rest of the body. A weak *sma-6(lf)* receptor allele gives a small phenotype without male tail defects characteristic of stronger *sma-6* alleles [8]. Overexpression of *dbl-1* results in long animals with only occasional tail defects, and the males are capable of mating [3, 9]. If saturating levels of signaling are achieved in the tail, then excess DBL-1 signaling above this threshold in *lon-2(lf)* animals may have little or no effect.

lon-2 Encodes a Novel Glypican Family Member

lon-2 alleles were originally isolated in a screen by Brenner [10]. We mapped and rescued the mutant phenotype

Table 1. *lon-2(e678)* Is Suppressed by Ligand and Receptor Mutant Alleles

Genotype	Body Length (% WT ±95% c.l.)	n
Wild-type	100 ± 2	27
dbl-1(wk70);lon-2(e678)	56 ± 2	16
sma-6(wk7)	84 ± 3	33
sma-6(wk7);lon-2(e678)	72 ± 3	29

Table 2. *lon-2(lf)* Mutants Are Longer than the Wild-Type

Genotype	Body Length (% WT ±95% c.l.)	n
Wild-type	100 ± 2	27
gpn-1(ok377)	95 ± 2	34
lon-2(e678)	114 ± 2	29
lon-2(e405)	116 ± 3	23
lon-2(e434)	116 ± 2	39
lon-2(e955)	115 ± 2	45
lon-2(e2140)	126 ± 10	20
lon-2(n1630)	124 ± 10	15

of *lon-2* animals with a KpnI/BamHI fragment of F35B9 (data not shown). This cosmid fragment includes the entire coding region of C39E6.1, 3682 bp of its 5′ promoter sequence, and 3331 bp of its 3′ sequence. Mutations in C39E6.1 were identified in the DNA of many *lon-2* mutant strains (Figure S1 in the Supplemental Data available with this article online). Furthermore, expressing *lon-2* genomic or cDNA sequences driven from the *lon-2* promoter (3 kb of its 5′ sequence) rescued the mutant phenotype (Table 4 and data not shown).

LON-2 contains the motifs that define glypicans, a type of heparan sulfate proteoglycan (HSPG). It has an N-terminal signal sequence (predicted to be amino acids 1–18 by Ensembl, version 39, June 2006) [11], 14 cysteines, and a putative SGXG glycosaminoglycan attachment site (amino acids 442–445) (Figure S1). Glypicans are also processed at the C terminus, where a glycophosphoinositol (GPI) tag attaches the molecule to extracellular membranes. LON-2 has a predicted GPI anchor site at amino acid 489 [12]. *lon-2(e2140)* is a missense mutation that substitutes one of the 14 highly conserved cysteines to a tyrosine. The phenotype of *lon-2(e2140)* is indistinguishable from that of the deletion mutant *lon-2(e678)* and transposon insertion mutant *lon-2(n1630)* (Table 2), indicating that the cysteine residue is functionally important. The other *C. elegans* glypican, *gpn-1*, has no significant phenotypic effect on body size (Table 2). LON-2 also has an RGD motif (amino acids 348–350), which in other systems binds integrins and other extracellular proteins. This motif is not present in the *C. briggsae* LON-2 sequence (CBP09675), but human GPC1 does contain an RGD motif immediately after its N-terminal signal sequence. We mutated the LON-2 RGD site to RGA, a substitution known to destroy the integrin-binding ability of RGD-containing proteins [13, 14]. This construct rescues *lon-2(e678)* animals (Table 5),

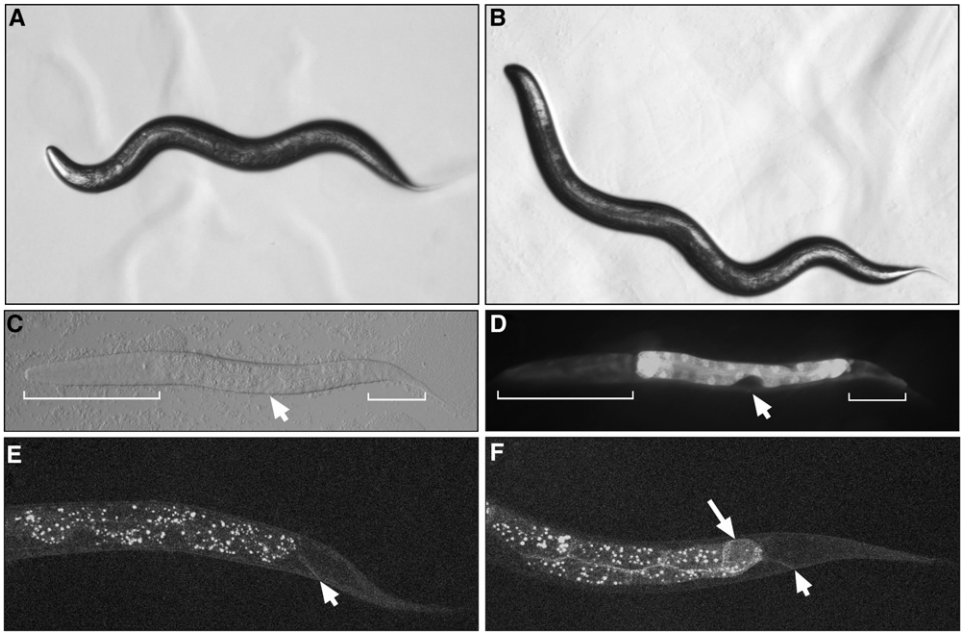

Figure 1. The *lon-2* Phenotype, Expression Pattern, and Localization

(A and B) *lon-2* mutant animals are longer in length than the wild-type. Animals are representative 24 hr adults. The anterior is left; dorsal is up. (A) shows the wild-type adult hermaphrodite. (B) shows the *lon-2(e678)* adult hermaphrodite.

(C and D) Expression pattern of *lon-2*. A *lon-2* promoter fusion construct with *gfp* shows strong intestinal expression and weak expression in the hypodermis of the head, body, and tail. Animals are at the L1 stage. DIC optics view (A) and fluorescence view (B). The head and the tail are indicated by bars, and the arrows indicate the gonad.

(E and F) A rescuing translational-fusion construct of a full-length LON-2 to GFP is visible in intestinal cells, predominantly to the most posterior cells. Animals are at the L1 stage. The confocal image of a non-transgenic *lon-2(e678)* animal shows low levels of autofluorescence (E). The confocal image of a transgenic *lon-2(e678);wkEx87* shows fluorescence of LON-2:GFP lining intestinal cells (indicated by the long arrow) (F). The rectum is indicated with short arrows in (E) and (F).

Table 3. *lon-2(e678)* Shows Additivity with *lon-1(wk50)*

Genotype	Body Length (% WT ±95% c.l.)	n
Wild-type	100 ± 2	27
lon-1(wk50)	111 ± 5	17
lon-1(wk50);lon-2(e678)	123 ± 6	16

suggesting that an RGD-mediated interaction with extracellular proteins is not necessary for LON-2 function.

Because HSPGs are posttranslationally modified, we asked whether null mutations in genes encoding enzymes that process HSPGs affect body size in *C. elegans*. *hse-5*, *hst-2*, and *hst-6* encode the single *C. elegans* orthologs of glucuronyl C5-epimerase, 2O-sulfotransferase, and 6O-sulfotransferase, respectively [15]. Notably, we noticed that animals with a deletion in any one of these genes are smaller than the wild-type (Table 6). *lon-2* is epistatic to all three heparin-sulfate-modifying enzymes, although *hse-5(lf);lon-2(lf)* animals, having a wild-type length, are intermediate in size (Table 6). However, *hse-5(lf);lon-2(lf)* animals are also frequently unhealthy and uncoordinated, and body size may be a secondary effect of these phenotypes. *hse-5(lf)* animals are only mildly uncoordinated [15]. The epistasis results, in conjunction with single-mutant phenotypes, are consistent with a model of LON-2 heparan-sulfate modificaton by HSE-5, HST-2, and HST-6 negatively regulating LON-2 activity.

LON-2 Is Required in the Hypodermis

To understand *lon-2* function during development, we first examined the *lon-2* transcriptional expression pattern. *gfp* driven by the *lon-2* promoter (3 kb of its upstream sequence) was expressed strongly in the intestine, most prominently in the most anterior and posterior cells (Figures 1C and 1D). Expression in these regions was observed throughout development, beginning in the embryo and continuing into adulthood. Very weak fluorescence was also seen in hypodermal cells in the head and tail (Figures 1C and 1D).

We then asked where the LON-2 protein localized subcellularly by generating transgenic nematode strains expressing an internally tagged, in-frame GFP fusion protein of LON-2. GFP was inserted at amino acid 26, between the predicted N-terminal signal sequence and the first conserved cysteine residue (at amino acid 43). This construct rescues *lon-2(e678)* animals to the wild-type or small (data not shown). GFP-tagged LON-2-protein fluorescence is weak and localizes to the surface of intestinal cells, most visibly to the most posterior cells (Figures 1E and 1F). This observation is consistent with a GPI modification of LON-2 because GPI moieties link their proteins to the extracellular surface of cell membranes.

Table 4. *lon-2(e678)* Is Rescued by Wild-Type *lon-2* cDNA

Genotype	Body Length (% *e678* ±95% c.l.)	n
lon-2(e678)	100 ± 2	55
Wild-type	88 ± 2	27
lon-2(e678);wkEx72 [lon-2p::yk185c5]	78 ± 2	17
lon-2(e678);wkEx73 [lon-2p::yk185c5]	78 ± 2	32

Table 5. *lon-2* cDNA without a Putative Integrin-Binding Site Rescues *lon-2(e678)*

Genotype	Body Length (% *e678* ±95% c.l.)	n
lon-2(e678)	100 ± 4	24
lon-2(e678);wkEx75 [lon-2p::lon-2(RGD->RGA)]	74 ± 3	24

Executors of the DBL-1 signaling pathway (the receptor genes *sma-6* and *daf-4*, a Smad gene, *sma-3*) and a known target gene, *lon-1*, have a similar expression pattern as *lon-2*, with strong expression in intestine and weaker expression in the hypodermis. These genes' products have been shown to be required in the hypodermis for body-size regulation [4, 5, 8, 16–18]. We asked whether LON-2 is also required in the hypodermis to control body size by performing cell-type-specific rescue experiments of body size. We found that expression of *lon-2(+)* in intestine with the *elt-2* promoter had little to no rescue of body size, whereas expression of *lon-2(+)* in hypodermal cells by the *elt-3* promoter rescued *lon-2(e678)* animals to a small phenotype (Table 7) [4]. Another hypodermal-specific promoter, *rol-6p*, driving *lon-2(+)* expression did not confer rescue of the long phenotype. However, *elt-3* and *rol-6* are expressed at different times, and this may explain the difference in their ability to rescue the *lon-2(lf)* phenotype [19, 20]. These results indicate that LON-2 functions at the level of hypodermal cells, which receive the DBL-1 signal, to restrict pathway activity.

Interestingly, the *dbl-1* pathway has been linked to the nematode innate immune response [21, 22], and the intestine is a focus of this action [23]. Notably, the DBL-1 pathway genes *sma-6*, *daf-4*, *sma-3*, and *lon-1* are also expressed in the intestine [4, 5, 8, 16–18]. Based on these observations and the fact that *lon-2* is strongly expressed in the intestine, *lon-2* expression in this tissue may function to regulate DBL-1-mediated innate immune responses.

lon-2(lf) Animals Are Rescued by *Drosophila* Glypican Gene *dally*

Dally, a *Drosophila* glypican, exhibits low conservation to LON-2 throughout the length of the protein, and the glypican motifs are entirely conserved (Figure S1). *dally* mutations result in flies with abnormal cell-division patterning, a result of genetic interactions with *dpp* [24, 25], *wingless* [24, 26, 27], and *hedgehog* signaling pathways

Table 6. *lon-2* Is Epistatic to HSPG-Modifying Enzyme Mutants

Genotype	Body Length (% WT ±95% c.l.)	n
Wild-type	100 ± 3	25
hse-5(tm472)	84 ± 2	23
hse-5(tm472);lon-2(e678)	99 ± 3	35
hst-2(ok595)	84 ± 3	23
hst-2(ok595);lon-2(e678)	113 ± 3	32
hst-6(ok273)	88 ± 3	32
hst-6(ok273) lon-2(e678)	123 ± 3	30

Table 7. Hypodermal Expression of *lon-2(+)* Rescues *lon-2(e678)*

Genotype	Expressed in	Body Length (% WT or *e678* ±95% c.l.)	n
lon-2(e678)		100 ± 2	55
lon-2(e678);wkEx72 [lon-2p::lon-2]		78 ± 2	17
lon-2(e678);wkEx73 [lon-2p::lon-2]		78 ± 2	32
lon-2(e678);wkEx85 [elt-2p::lon-2]	Intestine	92 ± 2	26
lon-2(e678);wkEx86 [elt-2p::lon-2]	Intestine	98 ± 2	15
lon-2(e678);wkEx82 [elt-3p::lon-2]	Hypodermis	76 ± 3	41
lon-2(e678);wkEx79 [rol-6p::lon-2]	Hypodermis	94 ± 4	31
lon-2(e678);wkEx80 [rol-6p::lon-2]	Hypodermis	90 ± 3	23

[28, 29]. In mammals, GPC3 negatively regulates cell proliferation through BMP and FGF signaling [30]. Mutations in this gene lead to Simpson-Golabi-Behmel syndrome, an overgrowth syndrome associated with an increased risk of forming certain tumors [31]. We asked whether Dally could functionally substitute for LON-2 in *C. elegans*. We drove *Drosophila dally* cDNA transcription from the *lon-2* promoter in *lon-2(e678)* animals. Transgenic animals were rescued to the wild-type body size (Table 8), thereby showing functional conservation between highly diverged *Drosophila* and *C. elegans* glypicans.

Table 8. *lon-2(e678)* Is Rescued by *Drosophila* Dally cDNA

Genotype	Body Length (% WT or *e678* ±95% c.l.)	n
lon-2(e678)	100 ± 2	22
lon-2(e678);wkEx74 [lon-2p::dally]	85 ± 3	18

LON-2 Binds BMP2 Ligand

To understand mechanistically how LON-2 regulates DBL-1 signaling, we asked whether LON-2 directly binds DBL-1. Because GPI-linked proteins are often insoluble, we eliminated the LON-2 GPI-anchor sequence by constructing a KpnI site by site-directed mutagenesis (5′-GGTTCA-3′ to 5′-GGTACC-3′), removing the C-terminal 65 amino acids, and adding the LET-23 transmembrane domain and a C-terminal T7 epitope tag (LON-2TM). LON-2TM was cloned into a mammalian expression vector and used to transfect 293T cells. When LON-2 was expressed in mammalian cells, the major form of the protein detected by western-blot analysis was the 150 kDa form (M2, Figure 2B), which likely reflects LON-2 modified by N-linked glycosylation and

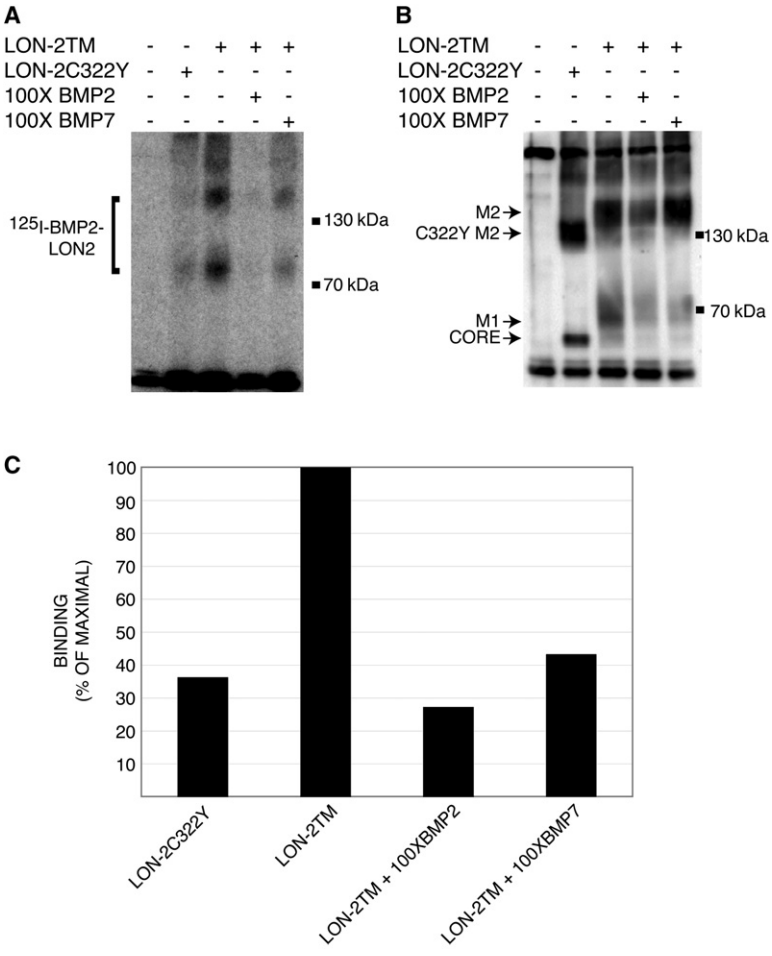

Figure 2. LON-2 Binds Directly to BMP2

(A and B) 293T cells were transiently transfected with an empty vector (Lane 1), LON-2TM(C322Y) (Lane 2), or LON-2TM (Lanes 3, 4, and 5) and incubated with 0.5 nM ^{125}I-BMP2. Specificity of LON-2 binding was assayed by competition with 50 nM (100× excess) unlabeled BMP2 (Lane 4) or BMP7 (Lane 5). Lysates were collected and immunoprecipitated with T7 antibody. Samples were split and separated on SDS-PAGE gels and exposed to film for visualization of ^{125}I-BMP2 (A) or immunoblotted with T7 antibody for visualization of LON-2TM (B). Three forms of the LON-2 protein were detected: a core protein at approximately 62 kDa, an N-glycosylated form at 70 kDa (M1), and a second 150 kDa (M2) modified form, which is N-glycolsylated and heparin-sulphate modified. The LON-2(C322Y) M2 band (C322Y M2) has a reduced size, consistent with a loss of N-glycosylation of the protein.

(C) ^{125}I signals were quantitated and normalized to LON-2TM protein expression levels.

glycosaminoglycans. This is consistent with previous studies, which revealed that Dally is modified by heparan sulfate [17]. We also observed low levels of a 62 kDa and a 70 kDa form of LON-2 (Core and M1, respectively, Figure 2B). The former is likely the unmodified protein core of LON-2, whereas the latter may reflect an N-glycosylated form of LON-2.

In the absence of readily available purified DBL-1, we used mammalian iodinated BMP2, which binds the *C. elegans* DBL-1 receptors SMA-6 and DAF-4 (data not shown and [32]), and asked whether it would bind to LON-2TM. We found that LON-2TM bound ^{125}I-BMP2 specifically, being efficiently competed by an excess of unlabeled BMP2, but less efficiently by BMP7 (binding is decreased to 27% or 43% of LON-2TM levels, respectively) (Figures 2A and 2C). Consistent with our results, it has recently been shown that *Drosophila* Dally binds BMP4 [33]. We also introduced the *lon-2(e2140)* mutation (C322Y) in the context of LON-2TM and asked whether it affected ligand binding. This mutation reduced BMP2 binding to 36% of the binding observed with LON-2TM (Figures 2A and 2C). The C322Y mutation also affected the posttranslational modification of the protein and resulted in a relative increase in the amount of the core protein observed and a loss of the 70 kDa modified form (Figure 2B). Consistent with a loss of the 70 kDa modification, there was a shift in the size of the heparin-sulfated form of LON-2(C322Y) (C322Y M2), although the amount of sulfated protein appeared to be similar (Figure 2B).

Taken together, these data indicate that LON-2 exerts its function in body-size control by modulating BMP signaling. We propose that LON-2 binds and acts as a reservoir for DBL-1, sequestering it and attenuating the strength of the signal in a dose-dependent manner. This is the first identification of a glypican acting on BMP signaling in *C. elegans* and establishes this model system for the study of extracellular regulation of growth-factor signaling. This is also the first case in which direct binding of BMP ligand to this family of HSPG has been shown in *C. elegans* and provides a basis for a more mechanistic understanding of glypican regulation. Further investigation of the mechanism of LON-2 action should provide insights into how the ligand/LON-2 complex interacts with receptors and affects signaling.

Supplemental Data

Supplemental Data include Experimental Procedures and one figure and can be found with this article online at http://www.current-biology.com/cgi/content/full/17/2/159/DC1/.

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